**Amendments to the Specification:**

**Please replace the paragraph on page 1, lines 13-31, with the following amended paragraph:**

This application is related to U.S. application Serial No. attorney dkt. no. 37851-922PC, entitled, "RATIONAL EVOLUTION OF CYTOKINES FOR HIGHER STABILITY, THE CYTOKINES AND ENCODING NUCLEIC ACID MOLECULES," to Rene Gantier, Thierry Guyon, Manuel Vega and Lila Drittanti. This application also is related to U.S. application Serial No. ~~Attorney docket no. 37851-923,~~10/658,355 filed the same day herewith, entitled "RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING," and to U.S. provisional application Serial No. 60/457,063, entitled "RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING," filed March 21, 2003, and to U.S. provisional application Serial No. 60/410,258, entitled "RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING," filed September 9, 2002, each to Rene Gantier, Thierry Guyon, Hugo Cruz Ramos, Manuel Vega and Lila Drittanti. This application also is related to co-pending U.S. application Serial No. 10/022,249, filed December 17, 2001, entitled "HIGH THROUGHPUT DIRECTED EVOLUTION BY RATIONAL MUTAGENESIS," to Manuel Vega and Lila Drittanti.

**Please replace the paragraph on page 4, lines 2-17, with the following amended paragraph:**

Provided herein are methods for directed evolution of families of proteins and resulting families of modified proteins. A family, such as the cytokine protein family, is initially identified. A property or phenotype for modification, such as resistance to proteolysis for increased stability in blood, is selected for modification. A representative member or members of the family, such as members of the ~~interfero~~ interferon $\alpha$ family, such as IFN$\alpha$-2b or IFN$\alpha$-2a, or interferon $\beta$ family, is (are) selected. It is modified using any directed evolution

method and protein(s) with a desired phenotype are screened and identified. In addition, the 3-dimensional structure of the protein can be mapped to topologically and spatially identify the loci that are modified to achieve the phenotypic change. 3-dimensional structures of other members of the family are generated or obtained and compared with the modified family member. Loci in the other family members that correspond on the protein to those modified in the original protein are identified and modified. The resulting proteins can be tested to confirm that they exhibit the modified phenotype.

**Please replace the paragraph on page 5, lines 7-19, with the following amended paragraph:**

Also provided herein are modified (mutant) cytokine proteins, such as variants of IFN$\beta$ and IFN$\alpha$, including IFN$\alpha$-2b and IFN$\alpha$-2a proteins and IFN$\beta$ proteins, that have altered, particularly, improved therapeutic properties, including higher stability compared to the unmodified forms. In particular, exemplary modified cytokines provided herein have increased stability, which, for example, improves their use as therapeutics. Among the modified cytokines provided herein are those that ~~exhibits~~ exhibit increased resistance to proteolysis compared to the unmodified cytokine. In particular, such resistance is at least 10%, 20%, 30%, 40%, 50%, 70%, 100% or more resistant to proteolysis compared to the unmodified cytokine. Also provided are cytokines that have increased anti-proliferative and/or antiviral activity and/or resistance to proteolysis compared to an unmodified cytokine.

**Please replace the paragraph on page 16, line 23, to page 17, line 8, with the following amended paragraph:**

As used herein, "is-HIT" refers to an *in silico* identified amino acid position along a target protein sequence that has been identified based on *i*) the particular protein properties to be evolved, *ii*) the protein's amino acid sequence, and/or *iii*) the known properties of the individual amino acids. These is-HIT loci

on the protein sequence are identified without use of experimental biological methods. For example, once the protein feature(s) to be optimized is (are) selected, diverse sources of information or previous knowledge (i.e., protein primary, secondary or tertiary structures, literature, patents) are exploited to determine those amino acid positions that may be amenable to improved protein fitness by replacement with a different amino acid. This step utilizes protein analysis "*in silico*." All possible candidate amino acid positions along a target ~~proteins~~ protein's primary sequence that might be involved in the feature being evolved are referred to herein as "*in silico* HITs" ("is-HITs"). The collection (library), of all is-HITs identified during this step represents the first dimension (target residue position) of the two-dimensional scanning methods provided herein.

**Please replace the paragraph on page 21, line 17, to page 22, line 3, with the following amended paragraph:**

As used herein, the phrase "unmodified target protein," "unmodified protein" or "unmodified cytokine," or grammatical variations thereof, refers to a starting protein that is selected for modification using the methods provided herein. The starting unmodified target protein can be the naturally occurring, wild type form of a protein. In addition, the starting unmodified target protein may have previously been altered or mutated, such that it differs from the native wild type isoform, but is nonetheless referred to herein as [[an]] a starting unmodified target protein relative to the subsequently modified proteins produced herein. Thus, existing proteins known in the art that have previously been modified to have a desired increase or decrease in a particular biological activity compared to an unmodified reference protein can be selected and used herein as the starting "unmodified target protein." For example, a protein that has been modified from its native form by one or more single amino acid changes and possesses either an increase or decrease in a desired activity, such as resistance to proteolysis, can be utilized with the methods provided herein as

the starting unmodified target protein for further modification of either the same or a different biological activity.

**Please replace the paragraph on page 27, lines 10-13, with the following amended paragraph:**

As used herein, treatment means any manner in which the symptoms of a condition, disorder or disease are ameliorated or otherwise beneficially altered. Treatment also encompasses any pharmaceutical use of the modified cytokines and compositions provided ~~herein~~ herein.

**Please replace the paragraph on page 29, lines 15-25, with the following amended paragraph:**

As used herein, nucleic acids include DNA, RNA and analogs thereof, including protein nucleic acids (PNA) and ~~mixture~~ mixtures thereof. Nucleic acids can be single or double stranded. When referring to probes or primers, optionally labeled, with a detectable label, such as a fluorescent or radiolabel, single-stranded molecules are contemplated. Such molecules are typically of a length such that they are statistically unique of low copy number (typically less than 5, generally less than 3) for probing or priming a library. Generally a probe or primer contains at least 14, 16 or 30 contiguous of sequence complementary to or identical a gene of interest. Probes and primers can be 10, 14, 16, 20, 30, 50, 100 or more nucleic acid bases long.

**Please replace the paragraph on page 41, lines 15-29, with the following amended paragraph:**

The 2-Dimensional rational scanning (or "2-dimensional scanning") methods for protein rational evolution provided herein (see, also copending U.S. application Serial No. ~~Attorney docket no. 923~~ 10/658,355, filed the same day herewith, based on U.S. provisional application Serial Nos. 60/457,063 and 60/410,258) are based on scanning over two dimensions. The first dimension

scanned is amino acid position along the protein sequence to identify is-HIT target positions, and the second dimension is the amino acid type selected for replacing a particular is-HIT amino acid position. An advantage of the 2-dimensional scanning methods provided herein is that at least one, and typically both, of the amino acid position scan and/or the replacing amino acid scan can be restricted such that fewer than all amino acids on the protein-backbone are selected for amino acid replacement; and/or fewer than all of the remaining 19 amino acids available to replace an original, such as native, amino acid are selected for replacement.

**Please replace the paragraph on page 43, lines 12-30, with the following amended paragraph:**

Provided herein is a method for directed evolution that includes identifying and selecting (using *in silico* analysis) specific amino acids and amino acid positions (referred to herein as is-HITs) along the protein sequence that are contemplated to be directly or indirectly involved in the feature being evolved. As noted, the 2-dimensional scanning methods provided include the following two-steps. The first step is an *in silico* search of a target protein's amino acid sequence to identify all possible amino acid positions that potentially can be targets for the activity being evolved. This is effected, for example, by assessing the effect of amino acid residues on the property(ies) to be altered on the protein, using any known standard software. The particulars of the *in silico* analysis is a function of the property to be modified. For example, in the example herein, a property that is altered <u>is</u> resistance of the protein to proteolysis.[[.]] To determine ~~aminoacid~~ <u>amino acid</u> residues that are potential targets as is-HITs, in this example, all possible target residues for proteases were first identified. The 3-dimensional structure of the protein was then considered in order to identify surface residues. Comparison of exposed residues with proteolytically cleavable residues yields residues that are targets for change.

**Please replace the paragraph on page 64, lines 8-15, with the following amended paragraph:**

Also provided are IFN$\alpha$-2b proteins that contain a plurality of mutations based on the LEADs (see, *e.g.*, Tables 6 and 7, EXAMPLE 5, which ~~listscandidate~~ lists candidate LEADs and LEAD sites), are generated. These IFN$\alpha$-2b proteins have activity that is further optimized. Examples of such proteins are described in the EXAMPLES. Other combinations of mutations can be prepared and tested as described herein to identify other LEADs of interest, particularly those that have further increased IFN$\alpha$-2b antiviral activity or further increased resistance to proteolysis.

**Please replace the paragraph on page 70, line 11 to page 71, line 22, with the following amended paragraph:**

Provided herein are methods for designing and generating new versions of native or modified cytokines, such as IFN$\alpha$-2b and IFN$\alpha$-2a. Using these methods, the redesigned cytokine maintains either sufficient, typically equal or improved levels of a selected phenotype, such as a biological activity, of the original protein, while at the same time its amino acid sequence is changed by replacement of up to: at least 1%, at least 2%, at least 3%, at least 4%, at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 12%, at least 14%, at least 16%, at least 18%, at least 20%, at least 30%, at least 40% up to 50% or more of its native amino acids by the appropriate pseudo-wild type amino acids. Pseudo-wild type amino acids are those amino acids such that when they replace an original, such as native, amino acid at a given position on the protein sequence, the resulting protein displays substantially the same levels of biological activity (or sufficient activity for its therapeutic or other use) compared to the original, such as native, protein. In other embodiments, pseudo-wild type amino acids are those amino acids such that when they replace an original, such as native, amino acid at a given position on the protein sequence, the resulting protein displays the same

phenotype, such as levels of biological activity, compared to an original, typically a native, protein. Pseudo-wild type amino acids and the appropriate replacing positions can be detected and identified by any analytical or predictive means; such as for example, by performing an ~~Alanine scanning~~ alanine scan. Any other amino acid, particularly another amino acid that has a neutral effect on structure, such as Gly or Ser, also can be used for the scan. All those replacements of original, such as native, amino acids by Ala that do not lead to the generation of a HIT (a protein that has lost the desired biological activity), have either led to the generation of a LEAD (a protein with increased biological activity); or the replacement by Ala will be a neutral replacement, i.e., the resulting protein will display comparable levels of biological activity compared to the original, such as native, protein. The methods provided herein for protein redesign of cytokines, such as IFN$\alpha$-2b and IFN$\alpha$-2a, are intended to design and generate "artificial" (versus naturally existing) proteins, such that they consist of amino acid sequences not existing in [[Nature]] nature, but that display biological activities characteristic of the original, such as native, protein. These redesigned proteins are contemplated herein to be useful for avoiding potential side effects that might otherwise exist in other forms of cytokines in treatment of disease. Other uses of redesigned proteins provided herein are to establish cross-talk between pathways triggered by different proteins; to facilitate structural biology by generating mutants that can be crystallized while maintaining activity; and to destroy an activity of a protein without changing a second activity or multiple additional activities.

**Please replace the paragraph on page 74, line 27, to page 75, line 6, with the following amended paragraph:**

In addition, the IFN$\alpha$-2b alanine scan revealed the following redesign-HITs having decreased antiviral activity at amino acid positions of IFN$\alpha$-2b corresponding to SEQ ID NO:1, amino acid residues: 2, 7, 8, 11, 13, 15, 16, 23, 26, 28, 29, 30, 31, 32, 33, 53, 69, 91, 93, 98, and 101. Accordingly, in

particular embodiments where it is desired to decrease the [[viral]] antiviral activity of IFN$\alpha$-2b or IFN$\alpha$-2a, either one or more of insertions, deletions and/or replacements of the native amino acid residue(s) can be carried out at one or more of amino acid positions of IFN$\alpha$-2b or IFN$\alpha$-2a corresponding to SEQ ID NO:1, amino acid residues: 2, 7, 8, 11, 13, 15, 16, 23, 26, 28, 29, 30, 31, 32, 33, 53, 69, 91, 93, 98, and 101.

**Please replace the paragraph on page 81, lines 20-31, with the following amended paragraph:**

Also provided herein is a method of structural homology analysis for comparing proteins regardless of their underlying amino acid sequences. For a subset of proteins families, such as the family of human cytokines, this information is rationally exploited herein. Human cytokines all share a common helix bundle fold, which is used to structurally define the 4-helical cytokine superfamily in the structural classification of the protein database SCOP© (Structural Classification of Proteins; see, e.g., Murzin *et al.*, *J. Mol. Biol.*, 247:536-540, 1995 and ~~"http://scop.mrc-lmb.cam.ac.uk/scop/").~~ scop.mrc-lmb.cam.ac.uk/scop/). This superfamily includes three different families: 1) the interferons/interleukin-10 protein family (SEQ ID NOS: 1 and 182-200); 2) the long-chain cytokine family (SEQ ID NOS: 210-217); and 3) the short-chain cytokine family (SEQ ID NOS: 201-209).

**Please replace the paragraph on page 86, lines 2-15, with the following amended paragraph:**

Also provided herein are modified cytokines or cytokine structural homologues of IFN$\alpha$-2b that are IFN$\alpha$ cytokines. These IFN$\alpha$ cytokines include, but are not limited to, IFN$\alpha$-2a, IFN$\alpha$-c, IFN$\alpha$-2c, IFN$\alpha$-d, IFN$\alpha$-5, IFN$\alpha$-6, IFN$\alpha$-4, IFN$\alpha$-4b, IFN$\alpha$-I, IFN$\alpha$-J, IFN$\alpha$-H, IFN$\alpha$-F, IFN$\alpha$-8 and IFN$\alpha$-consensus cytokine (see, SEQ ID No. 232). Accordingly, ~~amont the~~ among the modified IFN$\alpha$ cytokines provided herein are those with one or more amino acid replacements

at one or more target positions in either IFN$\alpha$-2a, IFN$\alpha$-c, IFN$\alpha$-2c, IFN$\alpha$-d, IFN$\alpha$-5, IFN$\alpha$-6, IFN$\alpha$-4, IFN$\alpha$-4b, IFN$\alpha$-I, IFN$\alpha$-J, IFN$\alpha$-H, IFN$\alpha$-F, IFN$\alpha$-8, or IFN$\alpha$-consensus cytokine corresponding to a structurally-related modified amino acid position within the 3-dimensional structure of the IFN$\alpha$-2b modified proteins provided ~~herein~~herein. The replacements lead to greater resistance to proteases, as assessed by incubation with a protease or a with a blood lysate or by incubation with serum, compared to the unmodified IFN alpha-2a.

**Please replace the paragraph on page 95, lines 4-9, with the following amended paragraph:**

In [[ther]] other embodiments, the modified cytokines provided herein possess increased activity compared to the unmodified cytokine. Stability can be assessed by any *in vitro* or *in vivo* method, such as by measuring residual inhibition of viral replication or to stimulation of cell proliferation in appropriate cells, after incubation with either mixtures of proteases, individual proteases, blood lysate or serum.

**Please replace the paragraph on page 95, line 18, to page 96, line 2, with the following amended paragraph:**

The 2D-scanning method and the 3D-scanning method (using structural homology) provided herein (see, copending U.S. application Serial No. 10/658,355, filed the same day herewith, based on U.S. provisional application Serial Nos. 60/457,063 and 60/410,258) were each applied to interferon $\beta$. Provided herein are mutant variants of the IFN$\beta$ protein that display improved stability as assessed by resistance to proteases (thereby possessing increased protein half-life) and at least comparable biological activity as assessed by antiviral or antiproliferation activity compared to the unmodified and wild type native IFN$\beta$ protein (SEQ ID NO: 196). The IFN$\beta$ mutant proteins provided herein confer a higher half-life and at least comparable biological activity with respect to the native sequence. Thus, the optimized IFN$\beta$ protein mutants

provided herein that possess increased resistance to proteolysis result in a decrease in the frequency of injections needed to maintain a sufficient drug level in serum, thus leading to, for example: *i*) higher comfort and acceptance by patients, *ii*) lower doses necessary to achieve comparable biological effects, and *iii*) as a consequence of (*ii*), likely attenuation of any secondary effects.

**Please replace the paragraph on page 96, line 28, to page 96, line 17, with the following amended paragraph:**

Two methodologies were used to address the improvements described above: (a) 2D-scanning methods were used to identify ~~aminoacid~~ amino acid changes that lead to improvement in protease resistance and to improvement in conformational stability, and (b) 3D-scanning, which employs structural homology methods ~~methods~~ also were used to identify ~~aminoaci~~ amino acid changes that lead to improvement in protease resistance. The 2D-scanning and 3D-scanning methods each were used to identify the amino acid changes on IFN$\beta$ that lead to an increase in stability when challenged either with proteases, human blood lysate or human serum. Increasing protein stability to proteases, human blood lysate or human serum is contemplated herein to provide a longer *in vivo* half-life for the particular protein molecules, and thus a reduction in the frequency of necessary injections into patients. The biological activities that have been measured for the IFN$\beta$ molecules are *i*) their capacity to inhibit virus replication when added to permissive cells previously infected with the appropriate virus, and *ii*) their capacity to stimulate cell proliferation when added to the appropriate cells. Prior to the measurement of biological activity, IFN$\beta$ molecules were challenged with proteases, human blood lysate or human serum during different incubation times. The biological activity measured, corresponds then to the residual biological activity following exposure to the proteolytic mixtures.

**Please replace the paragraph on page 98, lines 5-17, with the following amended paragraph:**

For the improvement of resistance to proteases, by 3D-scanning (structural homology):

1) Identifying some or all possible target sites (is-HITS) on the protein sequence that display an acceptable degree of structural homology around the ~~aminoacid~~ amino acid positions mutated in the LEAD molecules previously obtained for IFN$\alpha$ using 2D-scanning, and that are susceptible to digestion by one or more specific proteases; and

2) Identifying appropriate replacing amino acids, specific for each is-HIT, such that if used to replace one or more of the original amino acids at that specific is-HIT, they can be expected to increase the is-HIT's resistance to digestion by protease while at the same time, keeping the biological activity of the protein unchanged (these replacing amino acids are the candidate LEADs).

**Please replace the paragraph on page 121, lines 7-15, with the following amended paragraph:**

The optimized cytokine can be formulated for parenteral administration by injection e.g., by bolus injection or continuous infusion. Formulations for injection can be presented in unit dosage form e.g., in ~~ampoules~~ ampules or in multi-dose containers, with an added preservative. The compositions can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient can be in powder-lyophilized form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

**Please replace the paragraph on page 122, line 16, to page 123, line 17, with the following amended paragraph:**

Methods of treatment of cytokine-mediated or cytokine-involved diseases and immunotherapeutic methods are provided. The modified cytokines can be used in any method of treatment for which the unmodified cytokine is used. Hence the modified cytokines can be used for treatment of all disorders noted herein for the respective cytokines and for those known to those of skill in the art for each of the others, such as immunotherapeutic treatment (interleukins) and red blood cell expansion and stem cell expansion. The following table summarizes exemplary uses in addition to those noted herein of exemplary modified cytokines provided herein:

| **Cytokine** | **Exemplary Uses, Diseases and Treatment** |
|---|---|
| IL-10 | anti-inflammatory treatment of chronic liver injury and disease; myeloma |
| Interferon-gamma | interstitial/idiopathic pulmonary fibrosis; adjunctive immunotherapy for immunosupressed patients |
| Granulocyte colony stimulating factor | Crohn's disease; cardiac disease; acquired and congenital neutropenias; asthma |
| Leukemia inhibitory factor | myocardial infarction; multiple sclerosis; prevention of axonal atrophy;olfactory epithelium replacement stimulation |
| Human growth hormone | growth hormone deficiency; acromegaly |
| Ciliary neurotrophic factor | retinal degeneration treatments; neurodegnerative diseases such as Huntingtons; auditory degenerative diseases |
| Leptin | obesity; pancreatitis; endometreosis |
| Oncostatin M | chronic infammatory diseases; rheumatoid arthritis; multiple sclerosis; tissue damage supression |
| Interleukin-6 | protection from liver injury; Crohn's disease; hematopoietic associated diseases |
| Interleukin-12 | coksakievirus treatment;neuroblastoma; melanoma, renal cell carcinoma; mucosal immunity induction |
| Erythropoietin | hypoxia; myocardial ischemia; anemia with renal failure and cancer treatments |
| Granulocyte-macrophage colony stimulating factor | stimulate antigen presenting cells; anti-tumor activity for leukemia, melanoma, and breast, liver and renal cell carcinomas; adjunctive immunotherapy for immunosupressed patients; automimmune disease |

| Cytokine | Exemplary Uses, Diseases and Treatment |
|---|---|
| Interleukin-2 | immune reactivation after chemotherapy; melanoma; colon carcinoma |
| Interleukin-3 | leukemia cell targeting; motor neuropathy; amyotrophic lateral sclerosis; asthma |
| Interleukin-4 | allergic asthma; lupus |
| Interleukin-5 | treatment for parasites;asthma; allergic diseases accompanied by eosinophilia |
| Interleukin-13 | intracellular infections; B-cell cancers; asthma |
| Flt3 ligand | [[prostatate]] prostate cancer; myeloid leukemia; engraftment of allogenic hematopietic stem cells |
| Stem cell factor | hepatic injury; asthma; hematopoietic engraftment |

**Please replace the paragraph on page 127, line 22, to page 128, line 10, with the following amended paragraph:**

Lead mutants of Interferon alpha were first generated in the pSSV9-IFNa-EcoRI plasmid. With the only exception of E159H and E159Q, all mutants were amplified using the primers below. Primers contained NdeI (in Forward) and BamHI (in Reverse) restriction sites:

FOR-IFNA-5' AACATATGTGTGATCTGCCTCAAACCCACAGCCTGGGTAGC 3' (SEQ ID No. 1306; and

REV-IFNA-5' AAGGATCCTCATTCCTTACTTCTTAAACTTTCTTGCAAGTTTGTTG 3' (SEQ ID No. 1305)

Mutants E159H and E159Q were amplified using the following primers on reverse side (primer forward was the same than described above):

REV-IFNA-E159H-5' AAGGATCCTCATTCCTTACTTCTTAAACTGTGTTGCAAGTTTGTTG 3' SEQ ID No. 1304 above; and

REV-IFNA-E159Q-5' AAGGATCCTCATTCCTTACTTCTTAAACTCTGTTGCAAGTTTGTTG 3' SEQ ID No. 1305.

Mutants were amplified with Pfu Turbo Polymerase (Stratagene) ~~according~~. PCR products were cloned into pTOPO plasmid (Zero Blunt TOPO PCR cloning kit,

Invitrogen). The presence of the desired mutations was checked by automatic sequencing. The NdeI + BamHI fragment of the pTOPO-IFNa positive clones was then cloned into NdeI + BamHI sites of the pET11 plasmid.

**Please replace the paragraph on page 130, lines 5-16, with the following amended paragraph:**

IFN$\alpha$-2b mutants were produced in 293 human embryo kidney (HEK) cells (obtained from ATCC), using Dubelcco's modified Eagle's medium supplemented with glucose (4.5 g/L; Gibco-BRL) and fetal bovine serum (10%, Hyclone). Cells were transiently transfected with the plasmids encoding the IFN$\alpha$-2b mutants as follows: 0.6 x $10^5$ cells were seeded into 6 well-plates and grown for 36 h before ~~transfection~~transfection. Confluent cells at about [[70%,]]70% were supplemented with 2.5 $\mu$g of plasmid (IFN$\alpha$-2b mutants) and 10 mM poly-ethylene-imine (25 KDa PEI, Sigma-Aldrich). After gently shaking, cells were incubated for 16 h. Then, the culture medium was changed with 1 ml of fresh medium supplemented with 1% of serum. IFN$\alpha$-2b was measured on culture supernatants obtained 40 h after transfection and stored in aliquots at -80 °C until use.

**Please replace the paragraph on page 131, line 28, to page 132, line 3, with the following amended paragraph:**

Samples contained 1 mg of protein at 0.3 mg/ml (5 ml in total) in buffer. The GdnHCl ~~(Hydrochloride Guanidium)~~ (Guanidinium hydrochloride) present in the samples was eliminated by dialysis (minimum membrane cut = 10 kDa) overnight at 4°C against buffer ~~(1litre)~~(1 litre) (final concentration of GdnHCl : 43 [[Mm]] mM). Next, samples were further dialysed against ~~1litre~~ 1 litre of buffer during [[2:30h.]]2.5 hours. This step was repeated two additional times. After dialysis, very little precipitate was visible.

**Please replace the paragraph on page 134, lines 1-13, with the following amended paragraph:**

After 24 hours of growth, a 1/1000 EMC virus dilution solution was placed in each well except for the cell control row. Plates were returned to the $CO_2$ incubator for 48 hours. Then, the medium was aspirated and the cells were stained for 1 hour with 100 $\mu$l of Blue staining ~~solutio~~ solution to determine the proportion of intact cells. Plates were washed in a distilled water bath. The cell bound dye was extracted using 100 $\mu$l of ethylene-glycol mono-ethyl-ether (Sigma). The absorbance of the dye was measured using an Elisa plate reader (Spectramax). The antiviral activity of IFN $\alpha$-2b samples (expressed as number of IU/mg of proteins) was determined as the concentration needed for 50% protection of the cells against EMC virus-induced cytopathic effects. For proteolysis experiments, each point of for the kinetic measurements was assessed at 500 and 166 pg/ml in triplicate.

**Please replace the paragraph on page 135, line 27, to page 136, line 7, with the following amended paragraph:**

The percent of residual IFN $\alpha$-2b activity over time of exposure to proteases was evaluated by a kinetic study using either (a) 15 pg of chymotrypsin (10%wt/wt), (b) a lysate of human blood at dilution 1/100, (c) 1.5 pg of protease mixture, or (d) human serum. Incubation times were: 0 h, 0.5 h, 1 h, 4 h, 8 h, 16 h, 24 h and 48 h. Briefly, 20 $\mu$l of each proteolytic sample (proteases, serum, ~~bnlood~~ blood) was added to 100 $\mu$l of IFN $\alpha$-2b at 1500 pg/ml (500U/ml) and incubated for variable times, as indicated. At the appropriate time points, 10 $\mu$l of anti-proteases mixture, mini EDTA free, Roche (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/500) was added to each well in order to stop proteolysis reactions. Biological activity assays were then performed as described for each sample in order to determine the residual activity at each time point.

**Please replace the paragraph on page 136, lines 19-31, with the following amended paragraph:**

IFN$\alpha$ –2b mutants selected on the basis of their overall performance in vitro, were tested for pharmacokinetics in mice in order to have an indication of their half-life in blood in vivo. Mice were treated by subcutaneous (SC) injection with ~~aliquots~~ aliquots of each of a number of selected lead mutants. Blood was collected at increasing time points between 0.5 and 48 [[hs]]hours after injection. Inmediatedly after collection, 20 ml of anti-protease solution were added to each blood sample. Serum was obtained for further ~~analysis.Residual~~analysis. Residual IFN-$\alpha$ activity in blood was determined using the tests described in the precedent sections for in vitro characterization. Wild-type [[IFN $\alpha$]]IFN-$\alpha$ (that had been produced in bacteria under comparable conditions as the lead mutants) as well as a pegylated derivative of IFN $\alpha$, Pegasys (Roche), also were tested for pharmacokinetics in the same experiments.

**Please replace the paragraph on page 140, line 30, to page 141, line 5, with the following amended paragraph:**

The creation of N-glycosylation sites on the protein was a second strategy that was used to stabilize [[IFN$\alpha$-2b]]IFN$\alpha$-2b. Natural human IFN$\alpha$-2b contains a unique *O*-glycosylation site at position 129 (the numbering corresponds to the mature protein; SEQ ID NO:1), however, no *N*-glycosylation sites are found in this sequence. N-glycosylation sites are defined by the N-X-S or N-X-T consensus sequences. Glycosylation has been found to play a role in protein stability. For example, glycosylation has been found to increase bioavailability via higher metabolic stability and reduced clearance. In order to generate more stable IFN$\alpha$-2b variants, the N-glycosylation consensus sequences indicated above were introduced in the IFN$\alpha$-2b sequence by mutagenesis. Variants of IFN$\alpha$-2b carrying new glycosylation sites were assessed as previously described.

**Please replace the paragraph on page 149, 28, to page 150, line 2 with the following amended paragraph:**

The pSSV9 CMV 0.3 pA was cut by *Pvu*II and religated (this step gets rid of the ITR functions), prior to the introduction of a new *Eco*RI restriction site by Quickchange mutagenesis (Stratagene). The ~~oligonucleotides~~ oligonucleotide sequences used, follow:

EcoRI forward primer: 5'-GCCTGTATGATTTATTGGATGTTGGAATTCCCTGATGCGGTATTTTCTCCTTACG-3' (SEQ ID NO: 218)

EcoRI reverse prime: 5'-CGTAAGGAGAAAATACCGCATCAGGGAATTCCAACATCCAATAAATCATACAGGC-3' (SEQ ID NO: 219)

**Please replace the paragraph on page 151, lines 12-21, with the following amended paragraph:**

Two activities were measured directly on IFN samples: antiviral and antiproliferation activities. Dose (concentration) - response (activity) experiments for antiviral or antiproliferation activity allowed for the calculation of the 'potency' for antiviral and antiproliferation activities, respectively. Antiviral and antiproliferation activities also were measured after incubation with proteolytic samples such as specific proteases, mixtures of selected proteases, human serum or human blood. Assessment of activity following incubation with proteolytic samples allowed to determine the residual (antiviral or antiproliferation) activity [[an.d]] and the respective kinetics of half-life upon exposure to ~~proteases~~ proteases.

**Please replace the paragraph on page 151, lines 23-29, with the following amended paragraph:**

Antiviral activity of IFN $\beta$ was determined by the capacity of the cytokine to protect Hela cells against EMC (mouse encephalomyocarditis) virus-induced

cytopathic effects. The day before, Hela cells ($2x10^5$ cells/ml) were seeded in flat-bottomed 96-well plates containing 100 $\mu$l/well of Dulbecco's MEM-Glutamaxl-sodium pyruvate medium supplemented with 5% SVF and 0.2% of gentamicin. Cells were growth at 37°C in an atmosphere of 5% $CO_2$ for 24 [[hours]]hours.

**Please replace the paragraph on page 154, lines 1-11, with the following amended paragraph:**

The percent of residual IFN $\beta$ activity over time of exposure to proteases was evaluated by a kinetic study using 1.5 pg of protease mixture. Incubation times were: 0 h, 0.5 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h. Briefly, 20 $\mu$l of each proteolytic sample (proteases, serum, ~~bnlood~~ blood) was added to 100 $\mu$l of IFN $\beta$ at 400 and 800 pg/ml and incubated for variable times, as indicated. At the appropriate time points, 10 $\mu$l of anti-proteases mixture, mini EDTA free, Roche (one tablet was dissolved in 10 ml of DMEM and then diluted to 1/500) was added to each well in order to stop proteolysis reactions. Biological activity assays were then performed as described for each sample in order to determine the residual activity at each time point.